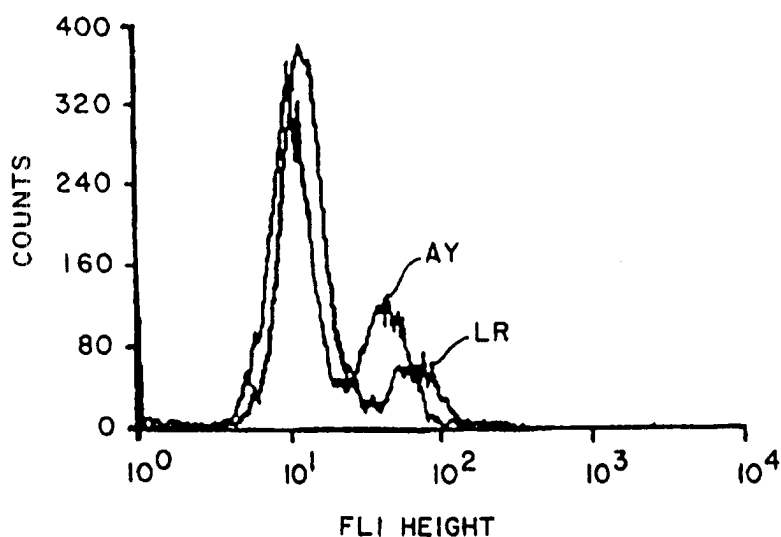




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(54) Title: FC RECEPTOR POLYMORPHISM



(57) Abstract

Methods are described for assessing the relative susceptibility of human patients to autoimmune diseases, bacterial and viral infections and tumors wherein the methods comprise identifying Fc γ RIIIA allelic patterns in human patients. Figure 1 is a graphic representation of the results of a flow cytometry experiment comparing the binding of IgG to blood cells from two patients having different Fc γ RIIIA allelic patterns.

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FC RECEPTOR POLYMORPHISM

10 Field of the Invention

This invention pertains to a polymorphism in the human gene for Fc γ RIIIA that affects the function of the encoded receptor. The invention further pertains to methods for assessing the relative susceptibility of human patient populations to autoimmune diseases, bacterial and viral infections, and tumors, and to methods for
15 gene therapy employing Fc γ RIIIA.

Background of the Invention

Fc receptors are membrane glycoproteins present on the surface of neutrophils, macrophages and other cell types, whose primary function is to bind and internalize
20 immunoglobulins, immune complexes, and immunoglobulin opsonized viruses, bacteria, and other particles. Three distinct families of human receptors for the Fc domain of immunoglobulin G (IgG) have been identified on the basis of reactivity with monoclonal antibodies, cellular distribution, and cDNA sequences: Fc γ RI, Fc γ RII, and Fc γ RIII. Within each of these three Fc γ receptor families, distinct
25 genes and alternative splice variants lead to a series of receptor isoforms that have striking differences in their extracellular, transmembrane, and intracellular regions. The salient features of the known classes of Fc γ receptors are compiled in Table 1.

Table 1

5	Structural isoforms	Fcγ Receptor Families						
		FcγRI			FcγRII			FcγRIII
	Distinct genes	A	B	C	A	B	C	A
	Splice variants		+	(+)	a1, a2	b1, b2, b3		
	Allelic variants	+	(+)	(+)	HR/LR	(+)		NA1 NA2
10	Membrane anchor	TM	TM	(TM)	a1:TM a2:secreted	TM	TM	γ/η/ζ complex GPI
Cell Distribution								
	Neutrophils	(+)	(?)		+		(?)	+
	Monocytes/MØ	+	(?)		+	(+) - 5% of donors	(?)	+
	Lymphocytes					B cells	(?)	NK cells
15	Mesangial cells of kidney							+

In addition to diversity based on distinct genes and their splice variants, different isoforms may also exhibit allelic polymorphisms. In several cases, the different alleles have been defined at the level of DNA sequence, and functional differences between the allelic forms have been noted. For example, the two recognized allelic forms of FcγRIIIb, NA1 and NA2, which differ by several amino acids and N-linked glycosylation sites, also differ in their capacity to mediate phagocytosis. In the case of FcγRIIA, the known allelic variants, HR ("high responder") and LR ("low responder"), which differ at amino acid position 131, differ substantially in their capacity to bind and internalize IgG2 (Salmon et al., 1992, *J. Clin. Invest.*, 89:1274). (In fact, FcγRIIA-LR is the only human FcγR that recognizes IgG2 efficiently.) Furthermore, allelic variants of FcγRI have also been found, though the possible functional significance of these sequence variations is not

yet clear. It is likely that more than two allelic forms exist for each Fc γ receptor gene.

The present inventors have identified a functionally significant allelic polymorphism in the Fc γ RIIIA gene, which results in the substitution of a valine for an phenylalanine at amino acid residue number 176 in the Fc γ RIIIA polypeptide (primary translation product). This amino acid substitution correlates with a differing ability of the two Fc γ RIIIA variants to bind IgG. Without wishing to be bound by theory, it is believed that the distribution of the Fc γ RIIIA isoform on monocytes, macrophages, mast cells, and, particularly, natural killer (NK) cells, as well as on kidney mesangial cells, means that this allelic polymorphism may affect the functioning of these cells in autoimmune diseases, tumor immunity, and antiviral and antibacterial immunity (and, in the case of mesangial cells, in mediating kidney disease). By contrast, an "expression polymorphism" was noted in one study that was interpreted as causing an apparent difference in the number (but not intrinsic binding capacity) of IgG binding sites on NK cells (Vance et al., *J.Immunol.* 151:6429, 1993).

Mononuclear phagocytes and NK cells lyse various target cell types via antibody-dependent cell-mediated cytotoxicity (ADCC). In ADCC, Fc γ RIIIA on the cytotoxic cell recognizes antibodies coating the surface of the target cells. Binding (and possibly aggregation) of Fc γ RIIIA then triggers synthesis and release of cytokines (such as, in the case of mononuclear phagocytes, interferon- γ , and in the case of NK cells, interleukin-2) as well as exocytic release of granules containing cytolytic compounds that effectuate lysis of the adjacent target cell. NK cells are also involved in lysis of some tumor cells and virally infected cells and participate in graft-versus-host disease (GVHD) reactions. Interestingly, occupancy of Fc γ RIIIA

on the surface of NK cells, coupled with exposure of the cells to interleukin-2, has also been shown to induce apoptosis (programmed cell death) in NK cells (Ortaldo et al., *J. Exp.Med.* **181**:339, 1995; Azzoni et al., *J. Immunol.* **154**:491, 1995).

Because of the important role played by Fc γ RIIIA-bearing cells in many types of immune responses, there is a need in the art for diagnostic tests that predict the relative capabilities of Fc γ RIIIA-bearing cells in an individual to participate in Fc γ RIIIA-mediated reactions. Furthermore, there is a need for reagents and methods for gene therapy to supplement the Fc γ RIIIA polypeptides present on an individual's immune cells with alternate allelic forms of Fc γ RIIIA that are more effective in immune surveillance, immune complex clearing, and other processes.

Summary of the Invention

The present invention encompasses methods for identifying the Fc γ RIIIA allelic pattern in a human patient, comprising obtaining genomic DNA from the patient and determining whether either or both copies of the Fc γ RIIIA gene contained therein comprises a pre-determined polymorphic DNA sequence. The determination may be achieved by: contacting the genomic DNA with an oligonucleotide specific for the pre-determined polymorphic DNA sequence, under conditions in which a hybrid can form between the oligonucleotide and its complement; and detecting any hybrid formed, wherein detection of a hybrid indicates the presence in at least one copy of the Fc γ RIIIA gene bearing the pre-determined polymorphic sequence. Alternatively, the determination may be achieved by purifying Fc γ RIIIA-expressing cells from a patient; extracting total RNA from the cells; and determining whether Fc γ RIIIA-encoding RNA comprises a pre-determined polymorphic sequence. In a preferred embodiment, the predetermined polymorphic sequence contains a G residue

at position 559 of the Fc γ R IIIA gene. (The numbering is from residue 1 of Fc γ R IIIA cDNA, in which, for example, the initiator methionine is encoded by residues 34-36).

In another aspect, the invention encompasses methods for establishing statistically significant correlations between the presence in human populations of one or more polymorphisms in Fc γ R IIIA alleles and (i) the incidence and severity of particular autoimmune diseases; (ii) the incidence of malignancies and clinical outcome; (iii) the incidence and clinical manifestations of different bacterial and viral diseases, including AIDS; and (iv) the efficacy of different types of vaccines in inducing protective immunity against particular pathogens, tumor antigens, or other immunogens. Any clinical circumstance in which NK cells and/or antibody-dependent NK cell reactions are involved is within the scope of the invention. Similarly, physiologically significant reactions involving surface Fc γ R IIIA polypeptides on monocytes/macrophages, on mast cells, on brain glial cells, and on kidney mesangial cells are also within the scope of the invention.

In one embodiment, the present invention provides diagnostic methods for determining predisposition to severe forms of a particular autoimmune disease in a patient, such as, for example, autoimmune glomerulonephritis, comprising establishing a statistically significant correlation between an Fc γ R IIIA allelic pattern and severity of the particular autoimmune disease; identifying the Fc γ R IIIA allelic pattern encoded by the patient's DNA; comparing the patient's allelic pattern with the corresponding Fc γ R IIIA allelic patterns in patients suffering from mild forms of the disease, severe forms of the disease, and no disease; and determining which of the corresponding patterns is most similar to the patient's Fc γ R IIIA allelic pattern.

In another embodiment, the present invention provides a diagnostic method for determining relative capacity in a patient producing anti-tumor antibodies for

mounting an effective Fc γ RIIIa-mediated tumor-killing response, comprising establishing a statistically significant correlation between an Fc γ RIIIa allelic pattern and incidence and clinical outcome of a particular malignancy; identifying the pattern of Fc γ RIIIa alleles encoded by the patient's DNA; comparing the pattern with the corresponding Fc γ RIIIa allelic patterns of humans with no malignancy, humans who experienced the particular malignancy and had a favorable clinical outcome, and humans who experienced the malignancy and had an unfavorable clinical outcome; and determining which of the corresponding patterns is most similar to the patient's Fc γ RIIIa allelic pattern.

In another embodiment, the invention provides a diagnostic method for determining the relative capacity in a patient producing antibodies directed against a viral or bacterial pathogen to mount an effective Fc γ RIIIa-mediated antibacterial or antiviral immune response, comprising establishing a statistically significant correlation between an Fc γ RIIIa allelic pattern and incidence and outcome of a particular bacterial or viral infection; identifying the pattern of Fc γ RIIIa alleles encoded by the patient's DNA; comparing the pattern with the corresponding Fc γ RIIIa allelic patterns of humans with no infection, humans who experienced the particular infection and had a favorable clinical outcome, and humans who experienced the particular infection and had an unfavorable clinical outcome; and determining which of the corresponding patterns is most similar to the patient's Fc γ RIIIa allelic pattern.

In another aspect, the invention provides methods for modifying the expression patterns of Fc γ RIIIa receptors in mononuclear phagocytes, NK cells, and/or kidney mesangial cells. These methods are carried out by using *in vivo* or *ex vivo* methods,

or combinations thereof, to introduce nucleic acids encoding alternate alleles of Fc γ R IIIA receptors into the appropriate recipient cells.

Brief Description of the Drawing

5 Figure 1 is a graphic representation of the results of a flow cytometry experiment which compared the binding of IgG to blood cells from two different patients. The X axis represents the fluorescence intensity exhibited by each cell, and the Y axis represents the number of cells in the sample that exhibited a particular fluorescence intensity. Independently, patient "AY" was shown to be homozygous for
10 the version of the Fc γ R IIIA gene that contains a T residue at position 559, while patient "LR" was shown to be homozygous for an alternate allelic version of the gene that contains a G residue at position 559.

Detailed Description of the Invention

15 All patent applications, patents, and literature references cited in this specification are hereby incorporated by reference in their entirety. In the case of inconsistencies, the present description, including definitions, will control.

Definitions:

1. "Allelic form" as used herein denotes an alternative version of a gene encoding the same functional protein but containing differences in its nucleotide sequence relative to another version of the same gene.

5 2. "Allelic polymorphism" or "allelic variant" as used herein denotes a variation in the nucleotide sequence within a gene, wherein different individuals in the general population may express different variants of the gene. A "predetermined polymorphic DNA sequence" is a known allelic variant.

10 3. "Allelic pattern" as used herein denotes the identity of each of the two copies of a particular gene in a patient, i.e., homozygosity for a particular allelic form, or heterozygosity encompassing two different allelic forms. The term "allelic pattern" is used interchangeably with "genotype".

15 4. "Amplification" of DNA as used herein denotes the use of polymerase chain reaction (PCR) to increase the concentration of a particular DNA sequence within a mixture of DNA sequences. For a description of PCR see Saiki et al., 1988, *Science*, 239:487.

20 5. "Chemical sequencing" of DNA denotes methods such as that of Maxam and Gilbert (Maxam-Gilbert sequencing, Maxam and Gilbert, 1977, *Proc. Natl. Acad. Sci. USA*, 74:560), in which DNA is randomly cleaved using individual base-specific reactions.

 6. "Enzymatic sequencing" of DNA denotes methods such as that of Sanger (Sanger et al., 1977, *Proc. Natl. Acad. Sci. USA*, 74:5463), in which a single-stranded DNA is copied and randomly terminated using DNA polymerase.

7. "Severe" autoimmune disease as used herein is defined as autoimmune disease encompassing organ-threatening and/or life-threatening symptoms. Severe autoimmune disease is often associated with clinical manifestations such as nephritis, vasculitis, or lung disease, or combinations thereof, that require aggressive treatment and may be associated with premature death. "Mild" autoimmune disease encompasses symptoms that may be function-altering and/or comfort-altering, but are neither immediately organ-threatening nor life-threatening.

8. An "effective Fc γ RIIIA-mediated immune response" as used herein is an immune response that results in the lessening or amelioration of at least one symptom of the pathological condition or syndrome to which the immune response is directed. A "favorable" clinical outcome for a particular condition is defined as one in which the patient's condition improved; while an "unfavorable" clinical outcome is one in which the patient's condition worsened.

The present invention provides methods for determining the allelic pattern of Fc γ RIIIA genes in human patients. The methods encompass the use of allele-specific oligonucleotides as hybridization probes and/or as primers for DNA amplification, as well as the use of direct DNA sequencing. Identification of receptor alleles may also be achieved immunologically, by contacting blood cells that express Fc γ RIIIA receptors on their cell surface with antibodies that distinguish between different polymorphic forms of the receptor. In a preferred embodiment, the invention provides a method for identifying the T/G polymorphism at position 559 in the Fc γ RIIIA gene.

The present invention is based on the finding that an allelic polymorphism in the gene encoding the human Fc γ RIIIA receptor isoform results in functionally distinct gene products. The "conventional" version of the DNA encoding Fc γ RIIIA

contains a T (thymine) at position 559; while the "polymorphic" version contains a G (guanine) at this position. (Nucleotide numbering is from position 1 in Fc γ R11A cDNA (Genbank Accession No. X52645 M31937). The substitution of G for T results in a change in the amino acid encoded at position 176 from phenylalanine to valine.

5 (The amino acid numbering is in reference to the primary translation product, i.e., residue 1 corresponds to the initiator methionine.) As described in more detail in Example 3 below, the Phe -> Val substitution correlates with a change in the capacity of the receptor to bind IgG. Consequently, it is believed that the expression of one or the other version of the receptor in an individual has important

10 consequences for the physiological activity of the receptor and thus for the functioning of the cell types that carry the receptor, i.e., monocytes/macrophages, NK cells, mast cells, and mesangial cells in the kidney.

The present invention provides a method for identifying the Fc γ R11A allelic pattern in human patients, which comprises testing blood cells or DNA from

15 individual patients for the presence of different Fc γ R11A allelic variants, using antibody-based and/or nucleic-acid-based methods which are described in more detail below. In a preferred embodiment, the method comprises determining whether each copy of the Fc γ R11A gene in the patient's DNA contains a T or a G residue at position 559 of the Fc γ R11A coding region and/or whether the Fc γ R11A

20 polypeptides expressed at the surface of immune cells of the patient contain the corresponding phenylalanine or valine residue at position 176.

The present invention also encompasses the identification and analysis of new allelic forms of Fc γ R11A genes, which can be achieved using methods well-known in the art, such as, for example, DNA sequencing (with or without prior amplification

25 of Fc γ R11A DNA segments); single-strand conformational polymorphism analysis

(SSCP) (Ravnik-Glavac et al., *Hum. Mol. Genet.*, 3:801, 1994); "HOT" cleavage (Cotton, et al., *Proc. Natl. Acad. Sci., USA*, 85:4397, 1988); denaturing gradient gel electrophoresis (DDGE) (Gulberg et al., *Nuc. Acids Res.*, 22:880, 1994); and combinations of the foregoing. Once a new polymorphism has been identified, immunological and/or molecular biological tests can be used to screen patients for the presence or absence of the polymorphism, as described for the T/G polymorphism at position 559. For example, monoclonal antibodies specific to the protein encoded by the newly identified allele can be prepared by well-known methods; these antibodies can then be used for screening of patient populations as described above. Alternatively, allele-specific oligonucleotides can be designed for use as probes and/or as primers in hybridization or PCR-based detection methods, respectively.

The invention also encompasses the establishment of statistically significant correlations, where they exist, between different allelic forms of Fc γ RIIIa (and allelic patterns formed by combinations of different alleles) and various physiological and/or clinical manifestations of variable Fc γ RIIIa function. For example, since Fc γ RIIIa is expressed on NK cells, monocyte/macrophages, mast cells, and mesangial cells in the kidney, polymorphisms that affect IgG binding by the receptor may affect any of the functions of these cells that are dependent on, and/or are initiated by, interaction of the receptor with antibody. Such functions include lysis of viruses and/or virally-infected cells; bacterial ingestion and killing; tumor cell killing; and reactions important in autoimmune diseases, such as, for example, clearing of immune complexes. Consequently, in practicing the present invention, correlations are sought between particular Fc γ RIIIa allelic forms and, for example: the risk of glomerulonephritis (mesangial cells and mononuclear phagocytes); the effectiveness of antibodies protective against viral infection or against tumor neo-

antigens (NK cells); and the risk of immune complex disease (mononuclear phagocytes).

Accordingly, studies are performed in which the incidence of disease caused by different viral and bacterial infections, as well as the clinical course and outcome, is correlated with particular Fc γ RIIIA allelic patterns. Similarly, the incidence of different types of malignancies (and clinical course and outcome) is correlated with particular Fc γ RIIIA allelic patterns. Finally, qualitative or quantitative aspects of particular autoimmune diseases, e.g., the number, severity, and duration of symptoms; the need for medication or other ameliorative treatment; and other clinical parameters, are also correlated with particular Fc γ RIIIA allelic patterns.

To establish these correlations, where they exist, the distribution of Fc γ RIIIA allelic patterns in a large number (several hundred) patients suffering from (or having a history of) viral or bacterial infection, malignancies, or autoimmune disease is determined using any of the methods described below, and compared with the distribution of Fc γ RIIIA allelic patterns in control (i.e., healthy) patients that have been matched for age and ethnic origin. A statistical method such as a 2x3 Chi square test is then used to determine whether the allele frequencies in the disease and normal groups are the same or different. In this manner, it is possible to obtain statistically significant correlations between a given pathological syndrome and Fc γ RIIIA alleles. It is believed that correlations between particular Fc γ RIIIA genotypes and particular diseases will provide an important prognosticator of disease susceptibility and clinical outcome.

Once a statistically significant correlation has been established between a clinically significant variable or symptom or susceptibility and a particular Fc γ RIIIA

allelic pattern, screening tests are devised to detect the particular allelic patterns in appropriate patient populations.

Determination of Fc γ R IIIA receptor genotypes according to the present invention may be performed in a susceptible population; alternatively, such testing can be performed after an initial diagnosis has been made. In this manner, different therapeutic interventions may be chosen for optimal long-term benefit. It will be understood that the particular Fc γ R IIIA allele that is screened for, the starting patient populations that are the targets of screening, and the test populations that provide the appropriate statistical database, will vary with the particular disease or syndrome.

In one case, if a given Fc γ R IIIA allele is rare, but is found to be strongly associated with a particular syndrome, large-scale screening may be appropriate if early therapeutic intervention can reduce or ameliorate later development of symptoms. For example, if a patient is found to express an Fc γ R IIIA allele that is associated with increased risk of particular complications, the patient might be treated prophylactically before the complications develop. Alternatively, a given Fc γ R IIIA allele may be common in the general population, and thus not be suitable for random screening. The same allele, however, when found in a patient already known to be suffering from a particular disease or syndrome, may correlate with the subsequent development of more severe manifestations of the disease. In this case, identification of a patient's Fc γ R IIIA genotype according to the present invention is performed after an initial diagnosis of the disease.

In practicing the present invention, the Fc γ R IIIA allelic pattern in an individual patient is determined by either: 1) immunological detection of one or more allelic forms of Fc γ R IIIA polypeptides present on the surface of appropriate immune cells ("phenotypic characterization"); or 2) molecular detection of the DNA or RNA

encoding one or more Fc γ R IIIA allelic forms using nucleic acid probes, with or without nucleic acid amplification or sequencing ("genotypic characterization").

In the first method, white blood cells or subsets thereof are isolated from a patient to be tested using methods that are well known in the art, such as, for example, gradient centrifugation and/or immunoadsorption. Antibodies that are capable of distinguishing between different allelic forms of Fc γ R IIIA are then applied to the isolated cells to determine the presence and relative amount of each allelic form. The antibodies may be polyclonal or monoclonal, preferably monoclonal. Measurement of specific antibody binding to cells may be accomplished by any known method, including without limitation quantitative flow cytometry, or enzyme-linked or fluorescence-linked immunoassay. The presence or absence of a particular allele, as well as the allelic pattern (i.e. homozygosity vs. heterozygosity) is determined by comparing the values obtained from the patient with norms established from populations of patients of known genotypes.

In an alternate embodiment, DNA is obtained from a patient, and the presence of DNA sequences corresponding to particular Fc γ receptor alleles is determined. The DNA may be obtained from any cell source or body fluid. Non-limiting examples of cell sources available in clinical practice include blood cells, buccal cells, cervicovaginal cells, epithelial cells from urine, fetal cells, or any cells present in tissue obtained by biopsy. Body fluids include blood, urine, cerebrospinal fluid, and tissue exudates at the site of infection or inflammation. DNA is extracted from the cell source or body fluid using any of the numerous methods that are standard in the art. It will be understood that the particular method used to extract DNA will depend on the nature of the source.

1 12. A diagnostic method for determining relative capacity in a patient
2 producing anti-tumor antibodies for mounting an effective Fc γ R IIIA -mediated tumor-
3 killing response, comprising

4 (i) establishing a statistically significant correlation between an
5 Fc γ R IIIA allelic pattern and incidence and clinical outcome of a particular
6 malignancy;

7 (ii) identifying the corresponding Fc γ R IIIA allelic pattern of said
8 patient;

9 (iii) comparing the Fc γ R IIIA allelic pattern of said patient with the
10 corresponding allelic patterns of humans with no malignancy, humans who
11 experienced the particular malignancy and had a favorable clinical outcome; and
12 humans who experienced the particular malignancy and had an unfavorable clinical
13 outcome; and

14 (iv) determining which of said corresponding allelic patterns is most
15 similar to the allelic pattern of said patient.

1 13. A diagnostic method as defined in claim 12, wherein said identifying
2 comprises

3 (a) obtaining genomic DNA from said patient, and

4 (b) determining whether either or both copies of the Fc γ R IIIA gene
5 contained in said genomic DNA comprises a pre-determined polymorphic DNA
6 sequence.

1 14. A diagnostic method as defined in claim 12, wherein said identifying
2 comprises

- 3 (a) purifying Fc γ R-expressing cells from said patient;
4 (b) extracting total RNA from said cells;
5 (c) determining whether Fc γ RIIIA-encoding RNA contained in said
6 total RNA comprises a pre-determined polymorphic RNA sequence.

1 15. A diagnostic method for determining relative capacity in a patient
2 producing antibodies directed against a viral or bacterial pathogen to mount an
3 effective Fc γ RIIIA-mediated antibacterial or antiviral immune response, comprising

4 (i) establishing a statistically significant correlation between an
5 Fc γ RIIIA allelic pattern and incidence and clinical outcome of a particular bacterial
6 or viral infection;

7 (ii) identifying the corresponding Fc γ RIIIA allelic pattern of said
8 patient;

9 (iii) comparing the Fc γ RIIIA allelic pattern of said patient with the
10 corresponding allelic patterns of humans with no infection, humans who experienced
11 the particular infection and had a favorable clinical outcome; and humans who
12 experienced the particular infection and had an unfavorable clinical outcome; and

13 (iv) determining which of said corresponding allelic patterns is most
14 similar to the allelic pattern of said patient.

1 5. A method for identifying the Fc γ RIIIA allelic pattern in a human
2 patient, said method comprising:

- 3 (i) purifying Fc γ R-expressing cells from said patient;
4 (ii) extracting total RNA from said cells;
5 (iii) determining whether Fc γ RIIIA-encoding RNA contained in said
6 total RNA comprises a pre-determined polymorphic RNA sequence.

1 6. A method as defined in claim 5, wherein said determining step
2 comprises subjecting said RNA to coupled reverse transcription-polymerase chain
3 reaction, under conditions in which RNA containing said pre-determined polymorphic
4 sequence is selectively transcribed and/or amplified.

1 7. A method as defined in claim 6, wherein said determining step further
2 comprises sequencing the products of said coupled reverse transcription-polymerase
3 chain reaction.

1 8. A method as defined in claim 5, wherein said polymorphic sequence
2 contains a G residue at position 559 of the Fc γ RIIIA gene.

1 9. A diagnostic method for determining predisposition to severe forms of
2 an autoimmune disease in a patient, comprising

- 3 (i) establishing a statistically significant correlation between an
4 Fc γ RIIIA allelic pattern and severity of said autoimmune disease;
5 (ii) identifying the corresponding Fc γ RIIIA allelic pattern of said
6 patient;

7 (iii) comparing the Fc γ RIIIA allelic pattern of said patient with the
8 corresponding allelic patterns of humans with no autoimmune disease, mild forms of
9 said autoimmune disease, and severe forms of said autoimmune disease; and

10 (iv) determining which of said corresponding allelic patterns is most
11 similar to the allelic pattern of said patient,

12 wherein if the Fc γ RIIIA allelic pattern of said patient is most similar
13 to the corresponding allelic pattern of human with severe forms of said autoimmune
14 disease, said patient has a predisposition to severe forms of said autoimmune disease.

1 10. A diagnostic method as defined in claim 9, wherein said identifying
2 comprises

3 (a) obtaining genomic DNA from said patient, and
4 (b) determining whether either or both copies of the Fc γ RIIIA gene
5 contained in said genomic DNA comprises a pre-determined polymorphic DNA
6 sequence.

1 11. A diagnostic method as defined in claim 9, wherein said identifying
2 comprises

3 (a) purifying Fc γ R-expressing cells from said patient;
4 (b) extracting total RNA from said cells;
5 (c) determining whether Fc γ RIIIA-encoding RNA contained in said
6 total RNA comprises a pre-determined polymorphic RNA sequence.

RESULTS: It is anticipated that cells bearing Fc γ RIIA allelic forms that bind IgG relatively less efficiently will also be less effective at ADCC.

Claims:

1. A method for identifying the Fc γ RIIIa allelic pattern in a human patient, said method comprising:

- (i) obtaining genomic DNA from said patient, and
- (ii) determining whether either or both copies of the Fc γ RIIIa gene contained in said genomic DNA comprises a pre-determined polymorphic DNA sequence.

2. A method as defined in claim 1, wherein the determining step comprises:

- (a) contacting said genomic DNA with an oligonucleotide specific for said pre-determined polymorphic DNA sequence, under conditions in which a hybrid can form between said oligonucleotide and its complement; and
 - (b) detecting any hybrid formed in step (a),
- wherein detection of said hybrid indicates the presence in at least one copy of the Fc γ RIIIa gene of said pre-determined polymorphic sequence.

3. A method as defined in claim 2, further comprising amplifying a region of the Fc γ RIIIa genes contained in said genomic DNA spanning said pre-determined polymorphic sequence prior to said contacting step.

4. A method as defined in claim 1, wherein said polymorphic sequence contains a G residue at position 559 of the Fc γ RIIIa gene.

1 16. A diagnostic method as defined in claim 15, wherein said identifying
2 comprises
3 (a) obtaining genomic DNA from said patient, and
4 (b) determining whether either or both copies of the Fc γ RIIIA gene
5 contained in said genomic DNA comprises a pre-determined polymorphic DNA
6 sequence.

1 17. A diagnostic method as defined in claim 15, wherein said identifying
2 comprises
3 (a) purifying Fc γ R-expressing cells from said patient;
4 (b) extracting total RNA from said cells;
5 (c) determining whether Fc γ RIIIA-encoding RNA contained in said
6 total RNA comprises a pre-determined polymorphic RNA sequence.

1 18. A diagnostic method for determining relative capacity in a patient to
2 mount an effective immune response to a vaccine, wherein said immune response is
3 mediated at least in part by Fc γ RIIIA, said method comprising
4 (i) establishing a statistically significant correlation between an
5 Fc γ RIIIA allelic pattern and the efficacy of the immune response to said vaccine;
6 (ii) identifying the corresponding Fc γ RIIIA allelic pattern of said
7 patient;
8 (iii) comparing the Fc γ RIIIA allelic pattern of said patient with the
9 corresponding allelic patterns of humans exhibiting an optimal immune response to
10 said vaccine and humans exhibiting a sub-optimal immune response to said vaccine;
11 and

- 12 (iv) determining which of said corresponding allelic patterns is most
13 similar to the allelic pattern of said patient.

1 19. A diagnostic method as defined in claim 18, wherein said identifying
2 comprises

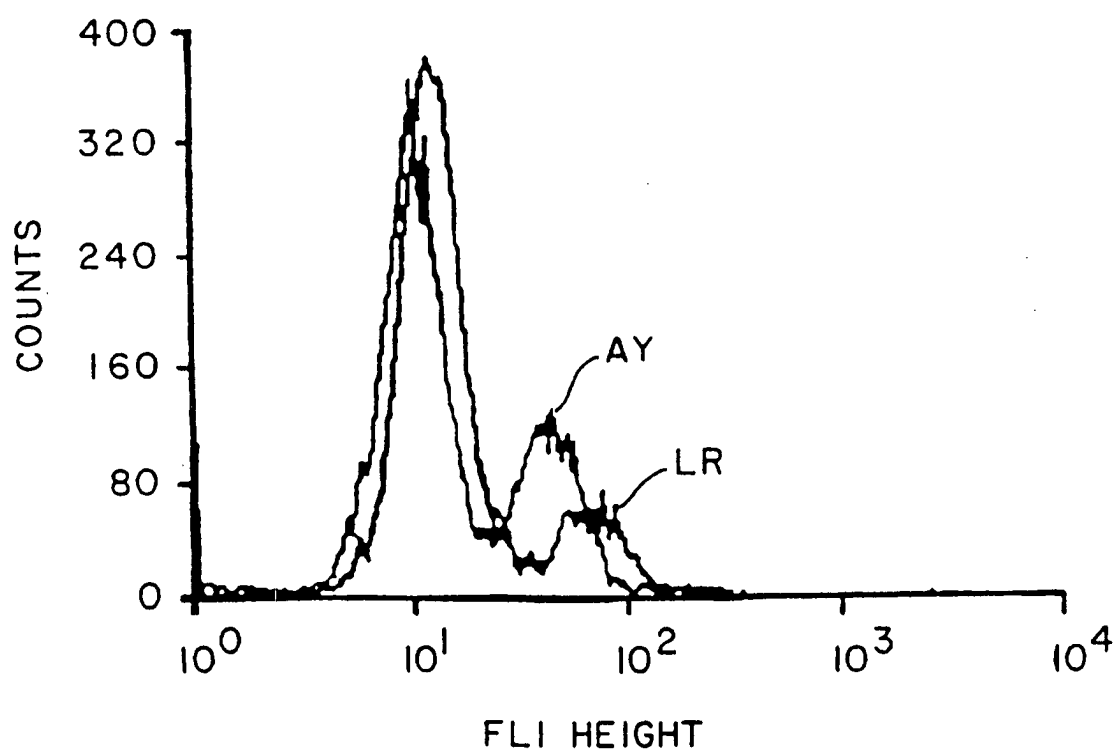
- 3 (a) obtaining genomic DNA from said patient, and
4 (b) determining whether either or both copies of the $Fc\gamma RIII A$ gene
5 contained in said genomic DNA comprises a pre-determined polymorphic DNA
6 sequence.

1 20. A diagnostic method as defined in claim 18, wherein said identifying
2 comprises

- 3 (a) purifying $Fc\gamma R$ -expressing cells from said patient;
4 (b) extracting total RNA from said cells;
5 (c) determining whether $Fc\gamma RIII A$ -encoding RNA contained in said
6 total RNA comprises a pre-determined polymorphic RNA sequence.

1/1

FIG. 1



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/11704

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12Q 1/68

US CL : 435/6

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DE HAAS et al. A Triallelic Fcγ Receptor Type IIIA Polymorphism Influences the Binding of Human IgG by NK Cell FcγRIIIa. The Journal of Immunology. April 1996, Vol. 156, pages 2948-2955, especially pages 2948, 2949 and 2955.	1-20
Y	RAVETCH et al. Alternative Membrane Forms of FcγRIII(CD16) on Human Natural Killer Cells and Neutrophils; Cell Type-Specific Expression of Two Genes that Differ in Single Nucleotide Substitutions. Journal of Experimental Medicine. August 1989, Vol. 170, pages 481-497, especially pages 484 and 488.	1-20

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
B earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z*	document member of the same patent family
O documents referring to an oral disclosure, use, exhibition or other means		
P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

10 SEPTEMBER 1997

Date of mailing of the international search report

24 SEP 1997

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks

Authorized officer

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US97/11704

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS; DIALOG; MEDLINE, CA, DERWENT PATENTS, BIOSIS, EMBASE, SCISEARCH
search terms: FcγRIIIA, CD16, polymorphism, mutation, allele, genotype

In an alternate embodiment, cells expressing Fc γ RIIIA are isolated by immunoadsorption, and RNA is isolated from the immunopurified cells using well-known methods such as guanidium thiocyanate-phenol-chloroform extraction (Chomocyznski et al., 1987, *Anal. Biochem.*, 162:156.) The isolated RNA is then
5 subjected to coupled reverse transcription and amplification by polymerase chain reaction (RT-PCR), using allele-specific oligonucleotide primers. Conditions for primer annealing are chosen to ensure specific reverse transcription and amplification; thus, the appearance of an amplification product is diagnostic of the presence of the allele specified by the particular primer employed. In another embodiment, RNA
10 encoding Fc γ RIIIA is reverse-transcribed and amplified in an allele-independent manner, after which the amplified Fc γ receptor-encoding cDNA is identified by hybridization to allele-specific oligonucleotides or by direct DNA sequencing.

Functional Implications of Fc γ RIIIA Allelic Polymorphism

15 As described in more detail in Example 3 below, the two allelic forms of Fc γ RIIIA that differ only in the presence of Phe or Val at position 176 differ markedly in their ability to bind IgG. IgG binding to Fc γ RIIIA is typically quantified by contacting Fc γ RIIIA-expressing cells with IgG that has been labelled. The labelled moiety may be labelled, for example, by fluorescence or radioactivity.
20 Alternatively, the primary IgG may be unlabelled, and a secondary, labelled antibody or other moiety may be used to detect bound primary IgG. The only condition is that the labelling procedure and/or presence of the label does not interfere with the receptor-ligand interaction. Following incubation, unbound IgG is removed by washing, and the amount of IgG bound to the cells is measured by any appropriate
25 means. As shown in Example 3, Fc γ RIIIA receptor polypeptides containing Val at

Once extracted, the DNA may be employed in the present invention without further manipulation. Alternatively, the DNA region corresponding to all or part of the Fc γ RIIIA may be amplified by PCR or other amplification methods known in the art. In this case, the amplified regions are specified by the choice of particular flanking sequences for use as primers. Amplification at this step provides the advantage of increasing the concentration of Fc γ RIIIA DNA sequences. The length of DNA sequence that can be amplified ranges from 80 bp to up to 30 kbp. Preferably, primers are used that define a relatively short segment containing sequences that differ between different allelic forms of the receptor.

The presence of Fc γ receptor allele-specific DNA sequences may be determined by any known method, including without limitation direct DNA sequencing, hybridization with allele-specific oligonucleotides, and single-stranded conformational polymorphism (SSCP). Direct sequencing may be accomplished by chemical sequencing, using the Maxam-Gilbert method, or by enzymatic sequencing, using the Sanger method. In the latter case, specific oligonucleotides are synthesized using standard methods and used as primers for the dideoxynucleotide sequencing reaction.

Preferably, DNA from a patient is subjected to amplification by polymerase chain reaction (PCR) using specific amplification primers, followed by hybridization with allele-specific oligonucleotides. Alternatively, SSCP analysis of the amplified DNA regions may be used to determine the allelic pattern. Most preferably, allele-specific PCR is used, in which allele-specific oligonucleotides are used as primers and the presence or absence of an amplification product indicates the presence or absence of a particular allele.

cytotoxicity (ADCC), using chicken erythrocytes (CE) coated with rabbit anti-CE IgG as target cells (see Example 4 below).

In another embodiment, the ability of different allelic forms of Fc γ RIIIA to support apoptosis of Fc γ RIIIA-expressing interleukin 2 (IL-2)-activated NK cells is quantified. In this assay, NK cells are first activated with IL-2 and then exposed to an IgG ligand capable of cross-linking cell-surface Fc γ RIIIA polypeptides. Cell death is then determined by methods well-known in the art, such as, for example, by visually monitoring trypan blue exclusion or by quantifying propidium iodide uptake by flow cytometry in combination with monoclonal antibodies directed against NK cell-surface components.

It will be understood that different allelic forms of Fc γ RIIIA may differentially affect the ability of NK cells to engage different effector mechanisms, such as, for example, lytic or homeostatic reactions (the former exemplified by tumor cell killing and the latter by regulation of NK cell populations). Similarly, different allelic forms of Fc γ RIIIA may differentially affect the ability of monocytes and macrophages to, e.g., clear immune complexes or trigger immune reactions. Furthermore, different allelic forms of Fc γ RIIIA may affect receptor-mediated intracellular signalling events such as [Ca⁺²]_i fluxes, and/or receptor-mediated increases in expression of HLA Class II molecules and interleukin-2 receptor molecules.

Applications

The present invention finds use in diagnostic and therapeutic methods that are based upon the existence of genetic polymorphisms in the Fc γ RIIIA gene that result in functional differences in the gene products.

Diagnostics

position 176 bind IgG more efficiently than wild-type (i.e., Phe₁₇₆-containing) receptors.

In one embodiment, binding of IgG to Fc γ RIIIA polypeptides is measured using purified cell populations that consist of, e.g., purified NK cells, purified
5 macrophages, and the like. In general, methods for cell purification are well-known in the art and may comprise without limitation: flow cytometry; density gradient centrifugation to obtain mononuclear cells; depletion of mononuclear phagocytes by adhesion to an appropriate solid support; negative selection (i.e., depletion) of different cell types by reaction with cell-type-specific monoclonal antibodies; and
10 combinations of the foregoing. Alternatively, multicolor flow cytometry can be used to identify a particular desired cell type (such as, e.g., NK cells) and to analyze IgG binding to that cell type, even without physical purification of the cells. The possible finding that an Fc γ RIIIA polymorphism selectively affects the functioning of particular Fc γ RIIIA-bearing cell types would allow the design of appropriate cell
15 type-specific therapeutic regimens.

In another embodiment, binding of IgG to Fc γ RIIIA polypeptides is measured using purified IgG subclasses, i.e., purified IgG1, IgG2, IgG3, and IgG4. The possible finding that a particular Fc γ RIIIA polymorphism affects receptor binding to a subset of IgG subclasses would be important in circumstances in which specific
20 antigens elicit antibodies that are restricted to particular subclasses.

The present invention also encompasses the identification of other functional consequences of the Phe \rightarrow Val substitution or of other allelic variations in the Fc γ RIIIA sequence. In one embodiment, cells expressing different allelic forms of Fc γ RIIIA are tested for their ability to mediate antibody-dependent cell-mediated

influenzae, and viral diseases such as those caused by influenza virus, adenoviruses, and human immunodeficiency virus.

Autoimmune diseases: The autoimmune diseases to which the methods of the present invention can be applied include without limitation systemic lupus erythematosus (SLE); systemic vasculitides such as Wegener's granulomatosis, polyarteritis nodosa, and cryoglobulinemic vasculitis; Sjogren's syndrome; mixed connective tissue disease; rheumatoid arthritis; and kidney diseases such as glomerulonephritis. The clinical manifestations of these diseases range from mild to severe. Mild autoimmune disease encompasses symptoms that may be function-
altering and/or comfort-altering, but are neither immediately organ-threatening nor life-threatening. Severe autoimmune disease encompasses autoimmune disease encompassing organ-threatening and/or life-threatening symptoms. Severe autoimmune disease is often associated with clinical manifestations such as nephritis, vasculitis, or lung disease, or combinations thereof, that require aggressive treatment and may be associated with premature death. Any statistically significant correlation that is found to exist between Fc γ RIIIA allelic patterns and any clinical parameters of autoimmune disease would enable the use of appropriate screening tests for Fc γ RIIIA allelic patterns as part of a diagnostic battery.

Vaccine efficacy: Individuals vary greatly in their response to different vaccines. In addition to differences in the level of antibodies elicited in different patients in response to a particular vaccine, the varying level of protection against the infectious agent may be due at least in part to a difference in the ability of Fc γ RIIIA-bearing immune cells to bind and/or lyse the infectious agent. Without wishing to be bound by theory, it is contemplated that even pre-existing test vaccines thought not to be effective (or to be only marginally effective) may in fact be effective only in

Cancer: Fc γ RIIIA-bearing cells such as NK cells and macrophages are thought to play an important role in immunological surveillance against developing malignancies. Accordingly, it is believed that, for certain types of malignancies at least, the statistical analyses described above will reveal statistically significant correlations between Fc γ RIIIA allelic patterns and at least one identifiable clinical parameter of cancer, such as, for example, survival time, response to chemotherapy or radiation therapy, extent of metastasis, and the like. If such correlations exist, appropriate screening tests for Fc γ RIIIA allelic patterns could be utilized to tailor therapeutic programs for each patient. These screening tests may be particularly applicable to patients in which treatment strategies are designed to evoke anti-tumor antibodies. Any tumor to which there is a host immune response involving antibodies that bind Fc γ RIIIA is within the scope of the invention. A non-limiting example of a malignancy to which the methods of the present invention can be applied is malignant melanoma.

Viral and bacterial infections: Different Fc γ RIIIA-bearing immune cells are thought to play important roles in combatting infection, macrophages play a primary role against bacterial infection and NK cells against viral infection. Any statistically significant correlation that is found to exist between Fc γ RIIIA allelic patterns and any clinical parameters of viral or bacterial infection, such as, e.g., susceptibility to infection, recurrence of infection, responsivity to antibiotics or antiviral agents, duration of infection, and the like, would enable the use of appropriate screening tests for Fc γ RIIIA allelic patterns as part of a diagnostic battery. Any infectious disease that evokes an immune response involving antibodies that bind Fc γ RIIIA is within the scope of the invention. Non-limiting examples include bacterial diseases such as those caused by *Streptococcus*, *Staphylococcus*, *Pneumococcus*, and *Hemophilus*

Therapeutics

The differential ability of different allelic forms of Fc γ R IIIA to bind IgG, and the corresponding differential response of patients bearing different Fc γ R IIIA allelic patterns to particular physiological stimuli means that it may be desirable to alter the Fc γ R IIIA expression patterns in patients for therapeutic purposes. This may be achieved by modifying Fc γ R IIIA expression levels (such as, for example, in mononuclear phagocytes by cytokine treatment with transforming growth factor- β or interferon- α) and/or by modifying the allelic forms of Fc γ R IIIA expressed in particular cells using gene transfer techniques. The phagocytic activity of macrophages, for example, makes them particularly suitable as recipients of Fc γ R IIIA -encoding nucleic acids.

The Fc γ R IIIA -encoding nucleic acids may be DNA or RNA, and include sense and antisense sequences. The nucleic acids may be flanked by their native regulatory sequences, or may be associated with heterologous sequences, including promoters, enhancers, response elements, signal sequences, polyadenylation sequences, introns, 5'- and 3'- noncoding regions, and the like. The nucleic acids may also be modified by many means known in the art. Non-limiting examples of such modifications include methylation, "caps", substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as, for example, those with uncharged linkages (e.g., methyl phosphonates, phosphotriesters, phosphoroamidates, carbamates, etc.) and with charged linkages (e.g., phosphorothioates, phosphorodithioates, etc.). Nucleic acids may contain one or more additional covalently linked moieties, such as, for example, proteins (e.g., nucleases, toxins, antibodies, signal peptides, poly-L-lysine, etc.), intercalators (e.g., acridine, psoralen, etc.), chelators (e.g., metals, radioactive metals, iron, oxidative

individuals carrying certain Fc γ RIIIA allelic patterns. It is also contemplated that patients whose Fc γ RIIIA polypeptides bind IgG less effectively may benefit from higher than conventional doses of immunogen (leading to higher threshold antibody levels) in order to achieve protective immunity. Accordingly, it is believed that in designing vaccine trials, stratifying patients by Fc γ RIIIA genotype would provide important information regarding vaccine efficacy.

B i o l o g i c s :

Therapeutic agents are currently being designed in which portions of the IgG Fc region are coupled to therapeutic moieties in order to prolong the *in vivo* half-life of the agents. For example, the monoclonal antibody OKT3 (Orthoclone), which is used clinically as an immunosuppressant, causes a variety of side effects (including Cytokine Release Syndrome). At least some of these side effects are likely to involve interaction of the administered antibody with Fc γ RIIIA receptors. (An example of Fc γ receptor-mediated side effects of an exogenously administered monoclonal antibody in a mouse system is described in Alegre et al., *J.Immunol.* 155:1544, 1995.) It is contemplated that the susceptibility of different patients to at least some of the side-effects of these agents may be correlated to their Fc γ RIIIA allelic patterns. Accordingly, pre-treatment screening of candidate patients using the methods of the present invention would provide an important benefit in achieving the most effective use of these agents.

Soluble forms of Fc γ RIIIA have also been reported to be cytotoxic for certain myeloma cells (Hoover et al., *J.Clin.Invest.* 95:241, 1995). It is contemplated that the differing ability of different allelic forms of Fc γ RIIIA to bind IgG according to the present invention will be an important influence on efficacy of the different allelic forms in adjunctive immunotherapy.

effects of a particular formulation are also determined by methods known in the art for each pathological condition or syndrome being treated. Any lessening or amelioration of any clinical signs or symptoms of a particular condition or syndrome that is achieved by administration of nucleic acids encoding an allelic variant of Fc γ RIIIA is within the scope of the invention.

The following working examples are intended to serve as non-limiting illustrations of the present invention.

Example 1: Determination of Fc γ RIIIA Allelic Patterns by DNA Amplification and Sequencing

The following studies were performed to identify allelic polymorphisms in the Fc γ RIII gene sequences.

Cell and RNA Isolation: Fresh anticoagulated human peripheral blood was separated by centrifugation through a discontinuous two-step Ficoll-Hypaque gradient (Salmon et al., 1990, *J. Clin. Invest.*, **85**:1287). Polymorphonuclear leukocytes (PMNs) were isolated from the lower interface and washed with HBSS (Gibco Laboratories, Grand Island, NY). Contaminating erythrocytes were lysed with hypotonic saline (0.02% NaCl) for 20 seconds followed by 0.16% NaCl and a final wash with HBSS. Mononuclear cells were isolated from the upper interface and washed with HBSS. RNA was then isolated from the mononuclear cell fraction using a phenol-guanidine thiocyanate-based procedure (TRIzol Reagent, Life Technologies, Gaithersburg MD).

RT-PCR Amplification of the Fc γ RIIIA coding sequence: Two sets of oligonucleotide primers were chosen to generate overlapping amplification products that together encompass the entire Fc γ RIIIA coding region. These primers are shown

metals, etc.), and alkylators. PNAs are also included. The nucleic acid may be derivatized by formation of a methyl or ethyl phosphotriester or an alkyl phosphoramidate linkage. Preferably, the sequences are present in appropriate nucleic acid vectors operably linked to regulatory sequences that allow their expression in the recipient cells. The expression may be transient or long-term, depending upon the expression system that is utilized. Any appropriate means for delivering the Fc γ RIIIa-encoding nucleic acids to the recipient cells may be used, including packaging in liposomes or recombinant viruses. Furthermore, both *in vivo* and *ex vivo* approaches may be used. For *in vivo* approaches, the nucleic acids are introduced directly into the patient; for *ex vivo* approaches, appropriate recipient cells are first removed from the patient and contacted with the Fc γ RIIIa-encoding nucleic acids under conditions appropriate for uptake and expression, after which the transformed cells are re-introduced into the patient:

Fc γ RIIIa-encoding nucleic acids may also be introduced into cells by recombination events, for example, by homologous recombination at the site of the endogenous gene or a sequence with substantial identity to the gene. Other recombination-based methods such as nonhomologous recombinations or deletion of endogenous genes by homologous recombination may also be used.

It will be understood that the particular vectors and delivery systems used in practicing the present invention may vary, depending upon the particular recipient cell type, the purpose for which treatment is performed, and other variables. For example, the amount of a particular vector, virus, or liposome preparation to be administered can be determined by experimentation known in the art, such as by establishing a matrix of dosages and frequencies and comparing a group of experimental units or subjects to each point in the matrix. Similarly, the therapeutic

are extended at 72°C for 10 min, after which the reaction is chilled at 4°C. The resulting amplified products are then analyzed by electrophoresis in 1.5% agarose gels, followed by staining with ethidium bromide, according to standard procedures.

DNA Sequencing: The PCR product described above is isolated from agarose gels using GeneClean II (Bio 101, La Jolla, CA), and subjected to automated cycle sequencing using ABI PRISM™ M13 dye primer kits and AmpliTaq™ DNA polymerase (Perkin-Elmer). DNA sequences are routinely determined from both strands, using sense or antisense primers, and reactions are analyzed on an ABI PRISM™ 377 automated sequencer (ABI/Perkin-Elmer).

RESULTS:

The following sequencing results were obtained from 29 individuals. The sequences were identical, except at position 559. At this position, 9 individuals were T/T homozygotes, 17 were T/G heterozygotes, and 3 were G/G homozygotes. All of these individuals had FcγRIIIA genes containing a T residue at position 230 (de Haas et al., *J. Immunol.* **156**:2948, 1996).

Example 2: Determination of FcγRIIIA Allelic Patterns Without Prior Cell Separation

The following approach is used to determine the FcγRIIIA allelic pattern in a patient, without the necessity for purifying mononuclear cells prior to analysis. This is achieved by using amplification primers that are specific for the FcγRIIIA gene. DNA is isolated from a patient from any available cell source.

(a) Nucleotides 486-640 of the FcγRIIIA gene are amplified using the following primers. (In each case, the sequence shown in bold is the sense or antisense

below. (In each case, the sequence shown in bold is the sense or antisense sequence corresponding to an Fc γ RIIA sequence, while the underlined sequence is an M13 sequence).

5 Set 1: sense primer

5'-CAGGAAACAGCTATGACCTCCCAACTGCTCTGCTACTT-3'

antisense primer

5'-TGTAAAACGACGGCCAGTCCTCAGGTGAATAGGGTCTTC-3'

10 Set 2: sense primer

5'-TGTAAAACGACGGCCAGTCCGGTG CAGCTAGAAGTCCA-3'

antisense primer

5'-CAGGAAACAGCTATGACCGGGGTTGCAAATCCAGAGAA-3'

Typically, 1-5 ng of RNA is incorporated into a 20 μ l reaction containing 0.5 μ g oligo(dT), 1 μ l of a mix containing 10mM of each deoxyribonucleotide triphosphate and 200 U reverse transcriptase in an RT-PCR reaction buffer (SuperScript Preamplification System, Gibco BRL). The reverse transcription reaction is carried out for 50 min at 42°C. The reaction is terminated and the RNA template is removed by addition of 2 U RNase H and incubation at 37°C for 20 min.

20 Then, the reaction is supplemented with 2 U of Taq DNA polymerase and deoxyribonucleotides to a final concentration of 200 nM. The following cycles of amplification are performed in a DNA Thermal Cycler (Perkin-Elmer Cetus, Norwalk, CT): Three cycles are performed consisting of 94°C, 3 min; 57°C, 45 sec; and 72°C, 1.5 min. Thirty cycles of amplification are then performed consisting of

25 94°C, 1 min; 59°C, 45 sec; and 72°C, 1.5 min. Finally, the amplification products

Example 3: IgG Binding to Different Fc γ RIIIA Allelic Forms

The following studies were performed to compare the ability of immune cells carrying different allelic forms of Fc γ RIIIA receptors to bind IgG.

Anticoagulated peripheral blood was obtained from each individual, and the
5 cells were washed with phosphate-buffered saline (PBS) to remove the plasma. The cells were incubated with 30 μ g/ml FITC-labelled polyclonal human IgG (Sigma Chemical Co.) for 30 min at 4°C, after which the unbound antibody was removed and the cells washed in PBS.

Bound IgG was quantified by flow cytometry using a FACScan (Becton
10 Dickinson Immunocytometry Systems, Mountain View, CA). For each experiment, the instrument was calibrated with standardized fluorescence microbead standards (Flow Cytometry Standards Corp., Research Triangle Park, NC) to allow assessment of both absolute and relative levels of immunofluorescence.

The results of a typical comparison between flow cytometry patterns obtained
15 from patients with different Fc γ RIIIA allelic patterns is shown in Figure 1. Cells obtained from Patient "LR", a G/G homozygote at position 559 of the Fc γ RIIIA gene, bind significantly more IgG on a per cell basis than those obtained from patient "AY", a "T/T homozygote at position 559 of the Fc γ RIIIA gene.

Table 2 shows the results of a study in which matched donor pairs from
20 patients having different Fc γ RIIIA allelic patterns at position 559 were compared with respect to IgG binding. In A, the samples were incubated with fluorescently-labelled IgG1; in B, the samples were incubated with unlabelled IgG1, followed by fluorescently-labelled anti-IgG; and in C, the samples were incubated with fluorescently-labelled pooled IgG. In parallel, each sample was incubated with a
25 monoclonal antibody (mAb) that recognizes Fc γ RIIIA irrespective of the amino acid

sequence corresponding to an Fc γ R IIIA sequence, while the underlined sequence is an M13 sequence).

sense primer:

5'-CAGGAAACAGCTATGACCTCATCATAATTCTGACTTCC-3'

5 antisense primer:

5'-TGTAAAACGACGGCCAGTCTTGAGTGATGGTGATGTTCA-3'

DNA is amplified and subjected to cycle sequencing as described in Example 1 above.

(b) In an alternative approach, allele-specific amplification is performed using

10 (i) a sense primer that corresponds precisely to an Fc γ R IIIA -specific sequence upstream of nucleotide 559 (as shown, nucleotides 486-505) and (ii) antisense primers that correspond either to the "T" allele or the "G" allele of Fc γ R IIIA at nucleotide 559. The rationale is that the pattern of amplification is indicative of the presence of either T or G at position 559. Representative primers are shown below:

15 sense primer:

5'-TCATCATAATTCTGACTTCC-3'

antisense primers:

5'-AAGACACATTTTTACTCCCAA-3' ("T" allele-specific)

5'-AAGACACATTTTTACTCCCAAC-3' ("G" allele-specific)

20 The primers are designed with intentional mismatches, if necessary, to optimize the allele-specific hybridization (and thus, allele-specific amplification) within a single reaction.

Example 4: Measurement of Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC)

The following studies are performed to compare the ability of immune cells carrying different allelic forms of Fc γ RIIIA receptors to mediate ADCC. The methods are essentially those described in Vance et al., *J. Immunol.* **151**:6429, 1993.

NK cells (effector cells) isolated as described in Example 1 above are resuspended in Medium 199 (M199) supplemented with 10% fetal bovine serum to give different effector:target ratios. To obtain fresh chicken erythrocyte (CE) target cells, wing vein blood is obtained on the day of the experiment, and the cells are washed three times in M199. 50 μ l of packed CE are then incubated in an additional 50 μ l of M199 and 50 μ l of ^{51}Cr (sodium chromate, DuPont-NEN, Boston MA) at 2 mCi/ml for 1 h at 37°C, after which the cells are washed twice with 10 ml M199 and resuspended at a concentration of 2.5×10^5 cells/ml.

50 μ l each of effector and labelled target cells and 50 μ l of anti-CE IgG are added to wells of a 96-well roundbottom plate and incubated for increasing times at 37°C in a humidified atmosphere containing 5% CO₂. 85 μ l aliquots of the supernatants are harvested and transferred to 2-mm glass tubes and counted in a gamma counter.

The percentage of cytotoxicity is calculated as $100 \times (\text{counts release with effectors} + \text{antibody}) - (\text{counts release with effectors alone}) / (\text{maximum lysis} - \text{spontaneous release})$. Maximum lysis is estimated by adding 50 μ l of labelled target cells to 100 μ l of a 20% SDS solution and harvesting 85 μ l of the supernatant. The nonspecific cytotoxicity is estimated by adding 50 μ l of labelled target cells to 100 μ l M199 and harvesting 85 μ l of the supernatant.